

LAP2 α and BAF collaborate to organize the Moloney murine leukemia virus preintegration complex

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Integration of viral DNA into the host genome is an essential step in retroviral replication. The viral DNA made by reverse transcription is a component of the preintegration complex (PIC) that also contains the viral integrase protein, the enzyme that integrates the viral DNA. Several other viral and cellular proteins are present in the PIC, but their functional roles are less well established. Barrier-to-autointegration factor (BAF) is a cellular protein component of the PIC that blocks autointegration of the viral DNA and stimulates intermolecular integration. In uninfected cells, BAF interacts with members of the LEM family of inner nuclear membrane and nucleoplasmic proteins. Here, we demonstrate that one of the LEM proteins, lamina-associated polypeptide 2 α (LAP2 α), is a component of the PIC. LAP2 α stabilizes the association of BAF with the PIC to stimulate intermolecular integration and suppress autointegration. To further understand the role of LAP2 α , we established LAP2 α -knockdown cell lines. Depletion of LAP2 α significantly inhibited viral replication. Our results demonstrate a critical contribution of LAP2 α to the nucleoprotein organization of the PIC and to viral replication.

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Introduction

DNA integration, an essential step for retroviruses to establish infection, is mediated by a large nucleoprotein complex derived from the core of the infecting virion (Goff, 1992; Brown, 1997; Asante-Appiah and Skalka, 1999; Craigie, 2001). This preintegration complex (PIC) is comprised of a copy of viral DNA, which is synthesized by reverse transcription of the viral RNA genome, and a number of viral and

cellular proteins. For Moloney murine leukemia virus (MoMLV), capsid protein, reverse transcriptase and integrase have been reported as viral protein components of the PIC (Bowerman *et al*, 1989; Fassati and Goff, 1999).

PICs isolated from virus-infected cells efficiently integrate their viral DNA into an exogenously added target DNA *in vitro* (intermolecular integration); on the other hand, intra-molecular integration into the viral DNA itself (autointegration) is avoided (Brown *et al*, 1987, 1989; Fujiwara and Mizuuchi, 1988; Bowerman *et al*, 1989; Lee and Craigie, 1994). This strong preference for intermolecular integration is a hallmark of the PIC. Autointegration would lead to destruction of viral DNA before it is able to migrate to the nucleus and integrate into chromosomal DNA. Barrier-to-autointegration factor (BAF) is a cellular component of MoMLV and human immunodeficiency virus type 1 (HIV-1) PICs, which blocks autointegration and stimulates intermolecular integration activity *in vitro* (Chen and Engelman, 1998; Lee and Craigie, 1998; Suzuki and Craigie, 2002; Lin and Engelman, 2003). This 89 amino-acid protein is highly conserved among multicellular eukaryotes with 60% sequence identity between human and *Caenorhabditis elegans* homologs (Cai *et al*, 1998). BAF exists as a dimer in solution (Cai *et al*, 1998) and binds double-stranded DNA, but not single-stranded DNA or RNA, with no detectable sequence specificity (Zheng *et al*, 2000). BAF bridges DNA molecules in a large nucleoprotein complex (Zheng *et al*, 2000), and we have proposed that the resulting compaction of the viral DNA within the PIC makes it inaccessible as a target for autointegration (Lee and Craigie, 1998; Suzuki and Craigie, 2002).

The function of BAF for the host cell is not well understood, but several lines of evidence point to a role in chromatin organization. BAF has profound effects on chromatin decondensation and nuclear growth in *Xenopus* extracts *in vitro* (Segura-Totten *et al*, 2002). It is associated with chromatin in a cell-cycle-dependent manner (Furukawa, 1999; Haraguchi *et al*, 2001; Furukawa *et al*, 2003) and the RNA interference (RNAi) phenotype in *C. elegans* revealed that knockdown of BAF caused a defect in chromatin segregation during mitosis (Zheng *et al*, 2000). In multicellular eukaryotes, BAF interacts with members of the LEM protein family (Lin *et al*, 2000), which includes Lamina-associated polypeptide 2 (LAP2), emerin and MAN1 proteins (Furukawa, 1999; Lin *et al*, 2000; Foisner, 2001; Lee *et al*, 2001; Shumaker *et al*, 2001). LEM proteins are components of the nuclear lamina structure at the nuclear periphery and of the lamin complexes in the nuclear interior (Foisner, 2001). This protein family is defined by a highly conserved motif of approximately 40 amino-acid residues near the N-terminus, the LEM domain (Lin *et al*, 2000), that directly interacts with BAF (Furukawa, 1999; Cai *et al*, 2001; Lee *et al*, 2001; Shumaker *et al*, 2001). As BAF binds to both DNA and LEM domain proteins, it has been proposed that BAF plays a key

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role in chromatin organization and may be involved in anchoring chromosomal DNA to the inner nuclear membrane (Furukawa, 1999; Dechat *et al*, 2000b; Shumaker *et al*, 2001; Furukawa *et al*, 2003; Segura-Totten and Wilson, 2004).

In murine cells, LAP2 and emerin have been identified as LEM proteins (Berger *et al*, 1996; Small *et al*, 1997). LAP2 is a well-characterized LEM protein. To date, six isoforms of the LAP2 have been identified in mammals; they are generated from one gene by alternative splicing (Harris *et al*, 1994; Furukawa *et al*, 1995; Berger *et al*, 1996). All of the isoforms share a conserved 186 amino-acid N-terminal domain (LAP2 common domain) and this common domain contains the LEM domain (Berger *et al*, 1996). Lamina-associated polypeptide 2 α (LAP2 α) is the largest isoform and, unlike most isoforms, it lacks a transmembrane region (Berger *et al*, 1996). Consequently, it is not membrane-bound and is localized predominantly throughout the nuclear interior, while a lesser amount has been shown to be present in the cytoplasm at stages of mitosis (Dechat *et al*, 1998; Vlcek *et al*, 1999).

We have previously demonstrated that BAF is a component of the MoMLV PICs that enhances intermolecular integration and blocks autointegration (Lee and Craigie, 1998; Suzuki and Craigie, 2002). The interaction of BAF with the LEM protein family gives rise to the new questions: (i) are any LEM proteins also associated with PICs? and (ii) if so, what is the role of the LEM proteins in the retroviral PIC and for infection? Here, we show that one of the LEM proteins, LAP2 α , is a component of MoMLV PIC and that LAP2 α stimulates the intermolecular integration activity of the PICs in collaboration with BAF. By analyzing the salt stability of a DNA complex with BAF and LAP2 α , and PICs from LAP2 α -knockdown cells, we find that the association of BAF with DNA is stabilized by LAP2 α . Furthermore, MoMLV replication is significantly inhibited in the LAP2 α -knockdown cells. Thus, our data suggest that LAP2 α plays an important role in the nucleoprotein organization of the PIC and in virus replication.

Results

LAP2 α associates with MoMLV PICs

To determine if any LEM proteins are associated with MoMLV PICs, murine LAP2 α (FLAG-LAP2 α), LAP2 β (FLAG-LAP2 β), LAP2 common domain (FLAG-LAP2c) and emerin (FLAG-emerin) proteins fused to the FLAG epitope (Figure 1A) were transiently expressed in NIH3T3 cells. Expression of the LEM proteins was confirmed by western blotting (Figure 1B). The transfected cells were cocultivated with the MoMLV-producing cell line clone 4 and cytoplasmic extract containing PICs was isolated. Anti-FLAG antibody immunoprecipitated the PICs derived from the FLAG-LAP2 α -expressing cells, but not PICs from the FLAG-LAP2 β - or FLAG-emerin-expressing cells (Figure 1B, lanes 1, 2 and 4 of the lower panel). Also, PICs derived from the FLAG-LAP2c-expressing cells were not recovered by immunoprecipitation with the anti-FLAG antibody (lane 3). These data suggest that only one of the LEM proteins, LAP2 α , is associated with PICs and the N-terminal LAP2 common domain alone is insufficient for this association.

To confirm the association of LAP2 α with the PICs, anti-LAP2 α polyclonal IgG was purified from a rabbit serum raised against hexahistidine (His)-tagged LAP2 α -specific domain (His-LAP2 α /AN186) and immunoprecipitation analysis with

this antibody was carried out on PICs from virus-infected NIH3T3 cells. PICs were efficiently recovered by immunoprecipitation with the anti-LAP2 α antibody, whereas immunoprecipitation with control rabbit IgG yielded very little recovery, demonstrating that endogenous LAP2 α is associated with PICs (Figure 2, upper panels); Western blotting controls with whole-cell lysates from NIH3T3 cells revealed that the anti-LAP2 α rabbit IgG specifically reacted with endogenous LAP2 α , but not with the other isoforms of LAP2 protein (Supplementary Figure S2). Interestingly, whereas stripping BAF from the PICs by high-salt treatment impaired the recovery of PICs by immunoprecipitation with anti-BAF serum as expected (Suzuki and Craigie, 2002), salt-stripped PICs were efficiently immunoprecipitated with anti-LAP2 α antibody (Figure 2, lower panels), indicating that LAP2 α remained associated with PICs after salt-stripping. We conclude that LAP2 α is a stable component of the MoMLV PIC and BAF is not essential for this association.

As a final test of the association of LAP2 α with the PIC, we incubated glutathione S transferase (GST) fusion recombi-

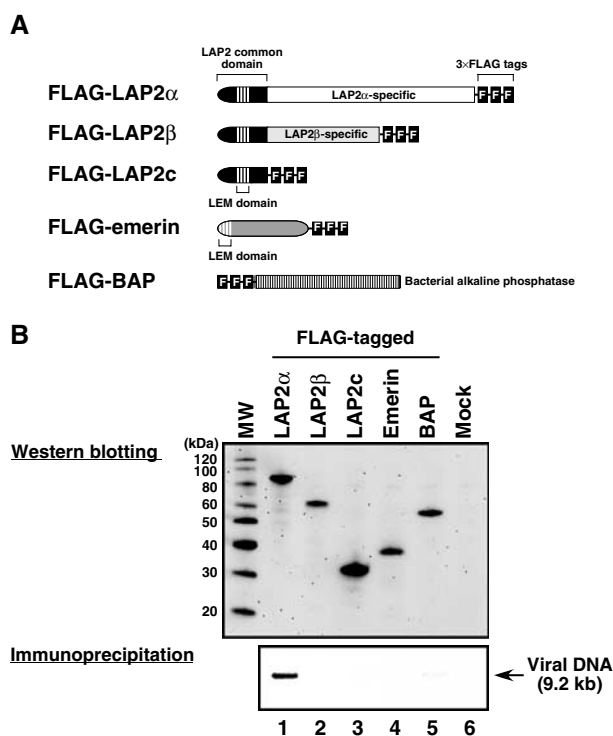


Figure 1 Immunoprecipitation of MoMLV PICs from FLAG-tagged LEM protein-expressing cells. **(A)** FLAG-tagged LEM protein constructs. **(B)** Expression of the FLAG-tagged LEM proteins in NIH3T3 cells and immunoprecipitation of PICs from these cells. NIH3T3 cells were transfected with the expression vectors encoding FLAG-tagged proteins and, at 24 h after transfection, the cells were cocultured with MoMLV-producing cells. Cytoplasmic extracts from these cells were immunoprecipitated with an anti-FLAG monoclonal antibody. The weak band in lane 5 of panel B is background that is not reproducibly observed. Viral DNA was extracted from the captured immunocomplex and detected by Southern blotting (lower panel). Protein expression in the cocultured cells was analyzed by western blotting using anti-FLAG monoclonal antibody (upper panel). MW: molecular weight marker. Control experiments demonstrated that the anti-FLAG antibody immunoprecipitates each of the LAP2 isoforms with similar efficiency (Supplementary Figure S1).

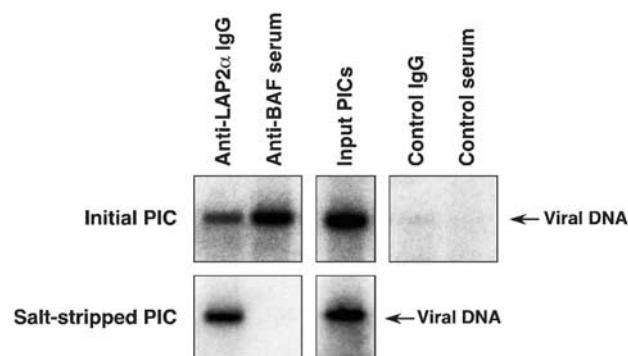


Figure 2 Association of endogenous LAP2 α with MoMLV PICs. Initial PICs (upper panels) and salt-stripped PICs (lower panels), made by coculture of NIH3T3 cells with MoMLV producer cells, were immunoprecipitated with the indicated antibodies and viral DNA was detected by Southern blotting.

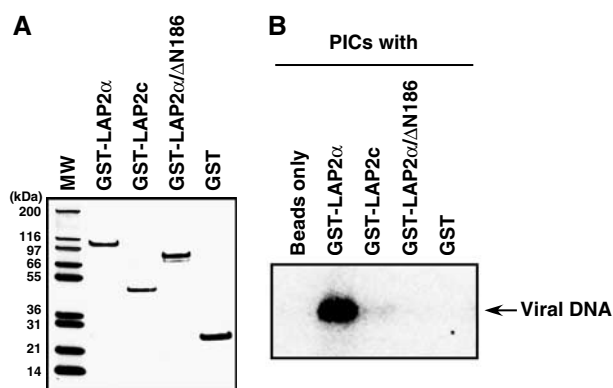


Figure 3 *In vitro* association of LAP2 α with MoMLV PICs. (A) PAGE of the purified GST-LAP2 α fusion proteins. (B) GST pulldown assay of PICs. Cytoplasmic extract containing PICs was incubated with the indicated GST-LAP2 α fusion proteins and precipitated by glutathione beads. The viral DNA in the bound fraction was detected by Southern blotting.

nant LAP2 α (GST-LAP2 α), LAP2 common domain (GST-LAP2c) and LAP2 α -specific domain (GST-LAP2 α /ΔN186) proteins (Figure 3A) with PICs and assayed for capture by glutathione beads. Incubation of PICs with GST-LAP2 α , but not with the truncated proteins or beads alone, resulted in recovery of PICs in this assay (Figure 3B). These data demonstrate that LAP2 α is able to associate with PICs *in vitro*, and suggests that both the LAP2 common and LAP2 α -specific domains are necessary for this association.

LAP2 α stimulates integration activity of PICs *in vitro*

To test if LAP2 α stimulates intermolecular integration, or blocks autointegration, we performed *in vitro* PIC integration assays in the presence of recombinant LAP2 α (Figure 4). Like BAF, His-LAP2 α stimulated the intermolecular integration activity of initial PICs (lanes 1–5); similar stimulation was also observed with GST-LAP2 α (data not shown). However, unlike the effect of BAF, stimulation of intermolecular integration by LAP2 α was not accompanied by a decrease in autointegration (compare lanes 4 and 5).

We next examined whether LAP2 α can restore the intermolecular integration activity of salt-stripped PICs. As ex-

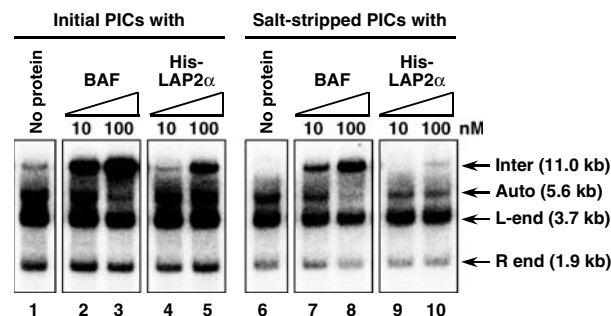


Figure 4 Stimulation of intermolecular integration of MoMLV PICs by LAP2 α . Initial PICs (lanes 1–5) and salt-stripped PICs (lanes 6–10) were incubated with BAF or His-LAP2 α and then added to the integration reaction mixture containing $\Phi \times 174$ RFI target DNA. After incubation, DNA products from the reaction were digested with *Bam*HI and detected by Southern blotting. The 11.0-kb band results from intermolecular integration of the viral DNA into $\Phi \times 174$ RFI DNA (inter). The 5.6-kb band and the smear below it result from autointegration of the viral DNA into itself (auto). The 3.7- and 1.9-kb bands are the unreacted viral DNA containing 5' LTR (L-end) or 3' LTR (R-end), respectively (Lee and Craigie, 1994).

pected, BAF efficiently restored the intermolecular integration preference and reduced the autointegration activity of salt-stripped PICs (Figure 4, lanes 6–8). In contrast, addition of His-LAP2 α (or GST-LAP2 α , data not shown) resulted in only minimal stimulation of intermolecular integration of salt-stripped PICs and did not suppress autointegration (Figure 4, lanes 9 and 10). Since immunoprecipitation of salt-stripped PICs shows that most of the BAF is dissociated by salt-stripping (Figure 2) (Suzuki and Craigie, 2002), this result suggests that the stimulation of intermolecular integration by LAP2 α requires BAF.

LAP2 α stabilizes association of BAF with DNA

A high salt concentration (greater than 500 mM KCl) is required to disrupt the protection of the PIC against autointegration (Lee and Craigie, 1994). At 400 mM KCl, PICs retain the ability to integrate intermolecularly, indicating that BAF remains associated with the PIC at a surprising high salt concentration (Figure 5A). We therefore tested whether the DNA-binding properties of BAF alone are sufficient to explain this behavior. We formed complexes of BAF with linearized $\Phi \times 174$ DNA in presence of 115 mM salt and then challenged these complexes with 150 or 400 mM salt concentration. The complexes were then sedimented in sucrose gradients containing 150 or 400 mM KCl, respectively, and the position of DNA in the gradient was monitored. Figure 5B shows that DNA alone remained in the top fractions of the gradient (panel 1), while the BAF/DNA complex sedimented faster in presence of 150 mM KCl (panel 2). However, in the presence of 400 mM KCl, the sedimentation rate was indistinguishable from that of free DNA, indicating that BAF was dissociated (Figure 5B, panel 3). The simple BAF/DNA complex is therefore unstable to high-salt challenge (Figure 5B, panel 3). We also tested if circular DNA could stabilize the BAF/DNA complex against the high-salt challenge, since both ends of viral DNA must be topologically closed by integrase within the PIC. However, the BAF/DNA complex with circular $\Phi \times 174$ DNA was also unstable to 400 mM KCl (Figure 5B, panels 4–6). These data indicate that, in contrast to the stable association of BAF with PIC, the complex of BAF with DNA

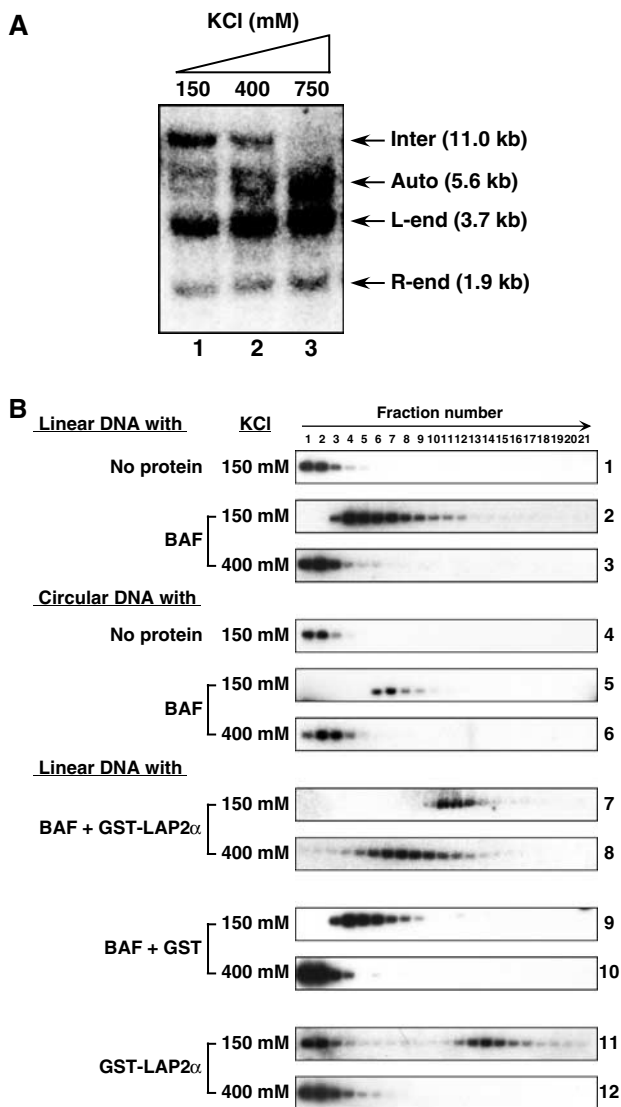


Figure 5 Stabilization of BAF/DNA complex by LAP2 α *in vitro*. (A) Retention of intermolecular integration activity of MoMLV PICs after treatment with 400 mM KCl. The PIC fractions were incubated in the presence of 150 mM (lane 1), 400 mM (lane 2), or 750 mM (lane 3) KCl and, after gel filtration, samples were added to integration reaction mixtures containing $\Phi \times 174$ RFI DNA. Products were digested with *Bam*HI and detected by Southern blotting. (B) Velocity sedimentation of BAF/DNA complexes with or without LAP2 α . BAF and LAP2 α were incubated with $\Phi \times 174$ DNA in buffer containing 115 mM KCl as indicated and then challenged with 150 or 400 mM KCl. After centrifugation in a sucrose gradient containing the same concentration of KCl, gradients were fractionated and the DNA was detected by Southern blotting.

alone is not stable to high salt and that the DNA-binding activity of BAF alone is insufficient to account for its incorporation into the PIC.

To determine if LAP2 α enhances the salt resistance of the association of BAF with DNA, we performed the sedimentation assay in the presence of GST-LAP2 α . The BAF/LAP2 α /DNA complex sedimented to near the middle of the gradient in the presence of 150 mM KCl (Figure 5B, panel 7). Surprisingly, even after treatment with 400 mM KCl, the complex did not dissociate and still sedimented much faster than free DNA (Figure 5B, panel 8). In control experiments, GST did not stabilize the BAF/DNA complex (Figure 5B,

panels 9 and 10). Interestingly, GST-LAP2 α alone was able to form a complex with DNA (Figure 5B, panel 11), although the affinity was lower than for BAF, as evidenced by free DNA at the top of the gradient. However, this GST-LAP2 α /DNA complex was also unstable at 400 mM KCl (Figure 5B, panel 12). These results demonstrate that BAF and LAP2 α together form a complex with DNA that is resistant to high salt.

Both the common and α -specific domains of LAP2 α are necessary for stabilization of the BAF/DNA complex

To determine the functional domains of LAP2 α that confer the stable association of BAF with DNA, we generated a series of deletion mutants lacking a part of the N- or C-terminal domain of LAP2 α as fusion proteins with GST and tested their ability to form a salt-resistant DNA complex with BAF and DNA (Figure 6). Both the LAP2 common domain, which contains LEM domain that interacts with BAF, and the LAP2 α -specific domain alone failed to stabilize the BAF/DNA complex in the presence of 400 mM KCl (Figure 6, panels 4 and 6). These data demonstrate that the common domain or α -specific domain alone is insufficient to form a stable complex with DNA and BAF. In the presence of 150 mM KCl, the LAP2 α deletion mutant missing the C-terminal half of the LAP2 α -specific domain (GST-LAP2 α /ΔC253) formed a complex with BAF/DNA that sedimented slightly faster than the complex of BAF alone with DNA (compare panel 2 of Figure 5 and panel 7 of Figure 6), but failed to form a salt-stable complex. In contrast, another deletion mutant (GST-LAP2 α /ΔN112), that contains the LEM domain and α -specific domain, but lacks the N-terminal 112 amino acids of common domain, did not alter the sedimentation behavior of the BAF/DNA complex (Figure 6, panel 9). These results suggest that, not only the LEM domain but also the N-terminal part of the common domain contributes to functional interactions with BAF and/or DNA. However, none of these deletion mutants were able to stabilize the BAF/DNA complex in the presence of 400 mM KCl (Figure 6, panels 8 and 10), demonstrating that the N-terminal part of the common domain and the C-terminal part of α -specific domain are necessary for the stabilization. We finally tested whether the independent proteins of the LAP2 common domain and LAP2 α -specific domain could together reconstitute the function of LAP2 α in stabilization of the BAF/DNA complex. Surprisingly, when the GST-LAP2c and GST-LAP2 α /ΔN186 were mixed together and incubated with BAF and DNA, the majority of the resulting complex was stable in the presence of 400 mM KCl (Figure 6, panels 11 and 12). We conclude that both the common domain and α -specific domains of LAP2 α are required to form a stable complex with BAF and DNA.

Salt stability of the PIC is diminished by *in vivo* depletion of LAP2 α

Velocity sedimentation of the complex of DNA with BAF and GST-LAP2 α revealed that association of BAF with DNA is reinforced by LAP2 α *in vitro* (Figure 5). Since the association of BAF with the PIC regulates the preference for intermolecular integration of the viral DNA (Suzuki and Craigie, 2002), depletion of LAP2 α would be expected to render the intermolecular integration preference more sensitive to salt challenge. Immunoprecipitation analysis of salt-stripped PICs using anti-LAP2 α serum reveals that LAP2 α is not efficiently

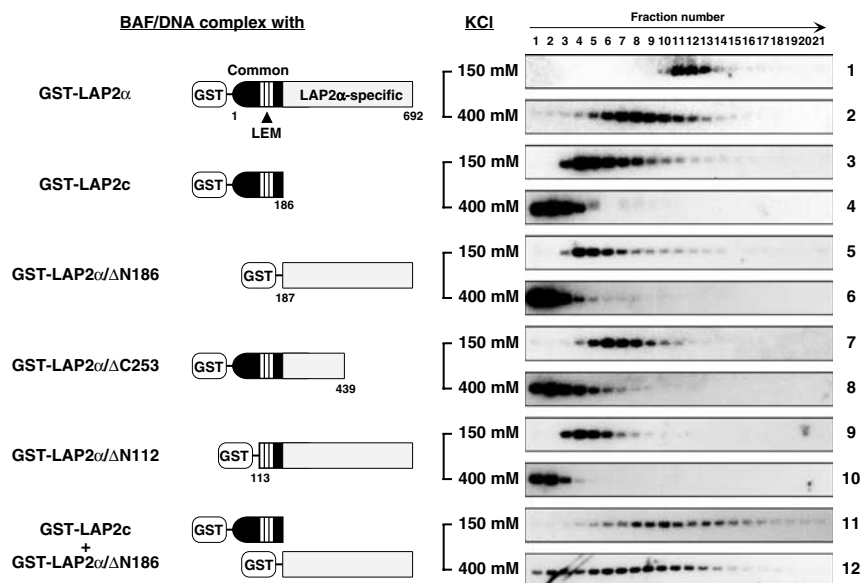


Figure 6 Stabilization of the BAF/DNA complex by LAP2 α requires both the LAP2 common and LAP2 α -specific domains. Complexes of DNA, BAF and deletion derivatives of LAP2 α were analyzed as in Figure 5B.

removed from the PIC by high-salt treatment (Figure 2). We therefore used the small interference RNA (siRNA) gene-silencing technique (Brummelkamp *et al*, 2002) to make a stable NIH3T3 cell line with substantially reduced levels of LAP2 α from which PICs were isolated (Figure 7). Growth of this cell line was indistinguishable from that of NIH3T3, although other cell lines with greater reductions in LAP2 α exhibited impaired growth (data not shown). We initially asked whether LAP2 α was depleted from PICs made in the LAP2 α -knockdown cells. Immunoprecipitation with anti-LAP2 α antibody shows that recovery of PICs derived from LAP2 α -knockdown cells was significantly decreased compared with PICs from NIH3T3 and siRNA control cells (Figure 7B). These data demonstrate that reduced levels of LAP2 α are associated with PICs from LAP2 α -knockdown cells.

We then examined whether the intermolecular integration activity of PICs from the LAP2 α -knockdown is more sensitive to salt challenge. PICs isolated from NIH3T3 cells, the knock-down cell line and control cell were treated with 400 or 750 mM KCl and assayed for integration activity. As predicted, although initial PICs from LAP2 α -knockdown cells were able to carry out intermolecular integration, this activity was mostly abolished after treatment with 400 mM KCl (Figure 7, lanes 4–6). In contrast, PICs from NIH3T3 and siRNA control cells still retained intermolecular integration activity after treatment with 400 mM KCl (Figure 7C, lanes 1–3 and 7–9).

Replication of MoMLV is inhibited in LAP2 α -knockdown cells

To evaluate the biological importance of LAP2 α in MoMLV replication, we tested whether the depletion of LAP2 α had an effect on virus replication. NIH3T3, LAP2 α -knockdown and siRNA control cells were infected with MoMLV for 2 h and, after washing, aliquots of culture supernatants were collected at 3, 6 and 9 days post infection and assayed for exogenous RT to monitor viral spread in the infected cultures. As shown in Figure 8, virus replication was drastically inhibited in LAP2 α -knockdown cells, but not in cultures of NIH3T3 and

siRNA control cells. The observed restriction of virus replication was the greatest when cells were infected with virus at low m.o.i. This demonstrates that LAP2 α also contributes to efficient replication of MoMLV.

Discussion

Chromosomal DNA, the normal target DNA for retroviral integration in infected cells, is enclosed by the nuclear membrane that forms the boundary between the nucleus and cytoplasm. Chromatin is attached to the inner nuclear membrane through a lamina structure, composed of lamin proteins and lamina-associated proteins, that is thought to be important for nuclear organization and function (Holaska *et al*, 2002). LEM proteins are the largest family of lamina-associated proteins. In the present study, we have reported that one of the LEM proteins, LAP2 α , is a stable component of MoMLV PIC. Furthermore, we have demonstrated a functional role for LAP2 α in the nucleoprotein organization of the PIC and for retroviral replication.

Why is only LAP2 α , and not other LEM proteins, associated with the PIC? LAP2 α is unique among LEM proteins in that it lacks a transmembrane domain and consequently is distributed throughout the nuclear interior rather than at the nuclear membrane (Dechat *et al*, 1998). Although LAP2 α is predominantly found within the nucleus, some LAP2 α also localizes in the cytoplasm, at least at the early stages of mitosis (Dechat *et al*, 1998; Vlcek *et al*, 1999). Indeed, when we checked the subcellular distribution of LAP2 isoforms by Western blotting of the pellet and supernatant fractions of NIH3T3 cells extracted with digitonin, a significant fraction of LAP2 α was observed in the cytoplasmic extract (data not shown). Therefore, it is likely that the presence of LAP2 α in cytoplasm enables its association to PIC, whereas the other isoforms sequestered by the nuclear envelope. However, the unique C-terminal domain of LAP2 α plays a crucial role in its recruitment since the FLAG-tagged common domain of LAP2 α , which binds BAF, did not associate with PICs.

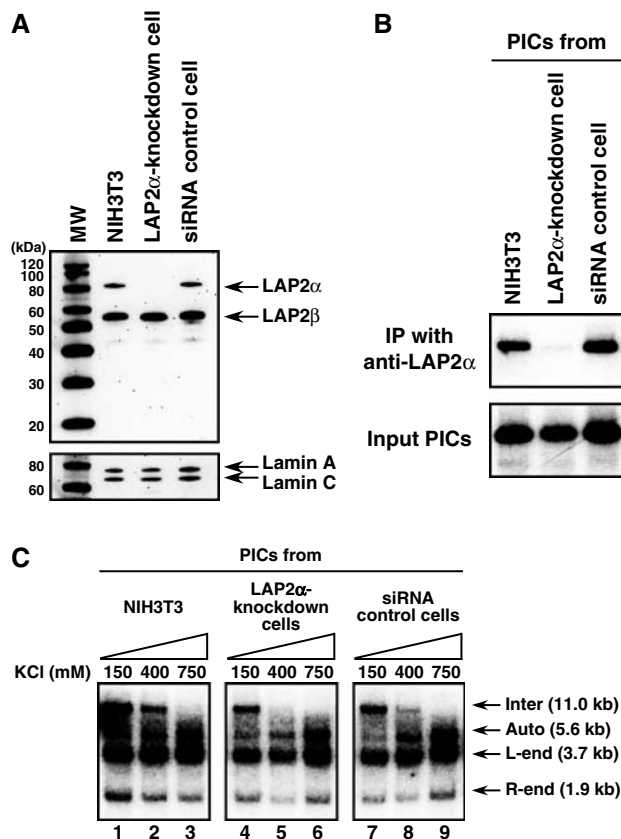


Figure 7 Depletion of LAP2 α *in vitro* renders MoMLV PICs more sensitive to high salt. (A) Establishment of LAP2 α -knockdown cells. NIH3T3 cells were transfected with a U6 promoter-driven siRNA expression vector encoding hairpin siRNA against LAP2 α or non-interacting siRNA (siRNA control), and stable cell lines were selected. Cell lysate from each cell line was subjected to western blotting analysis using anti-LAP2 common domain (upper panel) or anti-lamins A/C (lower panel) monoclonal antibodies. MW: molecular weight markers. (B) Reduced levels of LAP2 α are associated with MoMLV PICs from the LAP2 α -knockdown cell line. The PIC fractions from each cell line were immunoprecipitated (IP, upper panel) with anti-LAP2 α antibody and the recovered PICs were detected by Southern blotting. The lower panel shows the input PIC fractions without immunoprecipitation. (C) Diminished salt stability of PICs from the LAP2 α -knockdown cells. The PIC fractions from each cell line were treated with the indicated concentration of KCl and, after gel filtration, assayed for integration activity. Although there was some quantitative variation between experiments, with residual intermolecular integration activity sometimes being observed after treatment of PICs from NIH3T3 cells with 750 mM KCl, PICs that derived the LAP2 α -knockdown cells were consistently diminished in stability to 400 mM KCl.

How does LAP2 α promote the intermolecular integration activity of the PIC *in vitro*? Although His-LAP2 α stimulated intermolecular integration activity of initial PICs, when salt-stripped PICs were incubated with the His-LAP2 α , only minimal stimulation of intermolecular integration was observed (Figure 4). As immunoprecipitation with anti-BAF serum shows that most of the BAF is removed from the PIC by salt-stripping (Figure 2) (Suzuki and Craigie, 2002), these data indicate that LAP2 α requires BAF for the stimulation of integration activity. However, unlike stimulation of intermolecular integration by BAF, stimulation by LAP2 α does not appear to be accompanied by a decrease in autointegration.

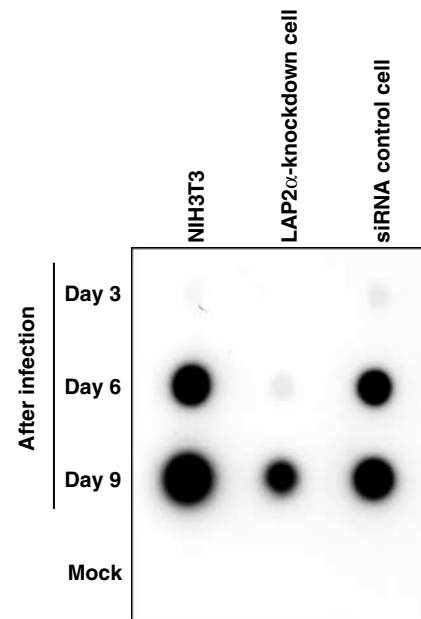


Figure 8 Inhibition of MoMLV replication in LAP2 α -knockdown cells. Each cell line was infected with MoMLV at low multiplicity for 2 h in the presence of polybrene and, after twice washing, cells were cultured in fresh culture medium. Culture supernatants were collected 3, 6, and 9 days after infection and virus spreading was monitored by RT activity assay.

An important clue as to the role of LAP2 α for the PIC is the stabilization of the BAF/DNA complex by LAP2 α . The difference in stability between the simple BAF/DNA complex and the association of BAF with the PIC suggests that the DNA-binding property of BAF alone is not sufficient for efficient incorporation into the PIC. Our results show that the salt resistance of the BAF/DNA complex was increased by the presence of LAP2 α , implying that LAP2 α may contribute to the efficient acquisition of BAF by the PIC. Shumaker *et al* (2001) have demonstrated that the LAP2 common domain prefers to bind BAF in a nucleoprotein complex with DNA rather than BAF alone. The structures of the BAF dimer, and the LEM and 'LEM-like' domains of the LAP2 common domain, have been reported (Cai *et al*, 1998, 2001; Umland *et al*, 2000). While the LEM domain directly interacts with BAF (Cai *et al*, 2001) and BAF binds DNA (Zheng *et al*, 2000), the interactions involving the other domains and DNA are unclear. The detailed structural organization of the complex of BAF, LAP2 α and DNA remains to be elucidated.

An important unanswered question remains as to how LAP2 α is retained in the PIC after salt-stripping? Most of the BAF is dissociated from the PIC by salt-stripping (Suzuki and Craigie, 2002). However, in the absence of BAF, the association of LAP2 α with DNA is unstable at 400 mM KCl. Therefore, unless a residual undetectable level of BAF in the salt-stripped PIC is sufficient to promote the association of LAP2 α , we speculate that another cellular and/or viral factor(s) may be involved in the stable association of LAP2 α with PIC. Preliminary immunoprecipitation experiments indicate that lamins A/C, which bind LAP2 α (Dechat *et al*, 2000a), appear not to be associated with MoMLV PICs (data not shown). The possible interactions between BAF and LAP2 α and other viral proteins present in the PIC need to

be investigated. Indeed, there is relatively little biochemical data on the interactions among the protein components of the PIC. Experiments are in progress to address this issue.

Finally, our data show that MoMLV replication is significantly inhibited in LAP2 α -knockdown cells, implicating a critical contribution of LAP2 α in virus infection. The step at which replication is inhibited in LAP2 α -knockdown cells remains to be determined; furthermore, the possibility that knockdown of LAP2 α indirectly affects viral replication should not be ignored. Unlike lentiviruses such as HIV-1, MoMLV can infect only dividing cells (Roe *et al*, 1993; Lewis and Emerman, 1994; Hatzioannou and Goff, 2001), because MoMLV PICs lack the appropriate nuclear localization signals to enter the intact nucleus of nondividing cells (Fouchier and Malim, 1999). Thus, it is commonly believed that MoMLV requires mitosis, during which the nuclear envelope is disassembled, for the PIC to access the chromosomal DNA. However, the molecular mechanisms for import of the PIC into the nucleus in dividing cells, and in nondividing cells, remain to be unambiguously established. Even if disassembly of the nuclear envelope during mitosis is a prerequisite for nuclear import of MoMLV PIC, why are PICs localized to the nuclear compartment when the envelope reforms? In the course of mitosis, the localization of LAP2 α in the cytoplasm and nucleus is dynamically changed (Dechat *et al*, 1998). In interphase and early stages of mitosis, LAP2 α is predominantly found in the nucleus. When disassembly of the nuclear envelope begins at metaphase and anaphase, LAP2 α is observed throughout the cell, without an apparent association with the chromosomal DNA. However, in the initial stage of nuclear reassembly at early telophase, LAP2 α redistributes to the nuclear interior (Dechat *et al*, 1998). Similarly, BAF has been shown to be diffusely non-localized at metaphase and anaphase, but specifically localized at chromatin core regions at telophase (Haraguchi *et al*, 2001). Thus, this re-localization of BAF and LAP2 α during the cell cycle may play a role in directing the PIC to the chromosomal DNA. In addition, these inner nuclear proteins may also contribute to the retention of the PIC within the nucleus in addition to blocking autointegration and stimulating inter-molecular integration. Therefore, it will be interesting to determine whether the nuclear entry step of MoMLV replication is blocked in the LAP2 α -knockdown cell lines. Determining the step(s) in which LAP2 α is involved during the virus replication cycle will be of help in understanding how retroviruses locate and direct integration to chromosomal DNA.

Materials and methods

Preparation of MoMLV PICs

NIH3T3 cells and the MoMLV-producing cell line clone 4 were grown in Dulbecco's modified Eagle medium containing high glucose, sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum (GibcoBRL). Standard preparation of MoMLV PICs was carried out as described previously (Fujiwara and Mizuuchi, 1988; Lee and Craigie, 1994). Cytoplasmic extract containing PICs was isolated with buffer A (20 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 150 mM KCl, 10 mM DTT) containing 20 μ g/ml aprotinin (Sigma) in the presence of 0.025% digitonin (Sigma) and stored at -80°C in buffer B (20 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 150 mM KCl, 10 mM DTT, 6 mM EDTA, 6% sucrose) until use (fraction I). In most experiments, fraction I was first subjected to gel filtration through a spin column of Sephacryl S-1000 Superfine

(Amersham-pharmacia) equilibrated with buffer B containing 0.1% BSA (fraction II).

High-salt treatment of PICs

For salt-stripping the PICs, fraction I was mixed with KCl to a final concentration of 750 mM and incubated on ice for 1 h. Salt-stripped PICs were separated from free components by gel filtration on a spin column of Sephacryl S-1000 equilibrated with buffer B containing 750 mM KCl and 0.1% BSA, and subjected to a second gel filtration on a spin column equilibrated with buffer B containing 0.1% BSA (Lee and Craigie, 1998). In some experiments, to test the salt stability of PICs, fraction I was also mixed with KCl to a final concentration of 400 mM KCl and subjected to gel filtration on a spin column equilibrated with buffer B containing 400 mM KCl and 0.1% BSA, followed by a second spin column equilibrated with buffer B containing 0.1% BSA.

Immunoprecipitation of PICs derived from FLAG-tagged protein-expressing cells

The cDNAs encoding full-length LAP2 α (residues 1–692), LAP2 β (residues 1–451), the LAP2 common domain (LAP2c, residues 1–186) and emerlin (residues 1–258) were amplified from a murine spleen cDNA library (Clontech) by PCR using *Pfu* polymerase (Stratagene) in the presence of 10% DMSO and cloned into the *EcoRV* site of p3XFLAG-CMV-14 mammalian expression vector (Sigma). In all, 20 μ g of the recombinant plasmid or control plasmid DNA, p3XFLAG-CMV-7-BAP (Sigma), was introduced into 1×10^6 of NIH3T3 cells by the calcium phosphate transfection method in a 10 cm diameter dish. At 24 h after transfection, the cells were washed once and cocultured with 5×10^5 of clone 4 cells in the presence of polybrene for 18 h. Cytoplasmic extract was prepared from infected cells using 500 μ l of buffer A with 0.025% digitonin and stored in buffer B as fraction I.

Fraction I was subjected to gel filtration through a spin column of Sephacryl S-1000 Superfine equilibrated with buffer B containing 0.1% BSA (fraction II), and 20 μ l of fraction II was incubated with 25 μ g of anti-FLAG monoclonal antibody M2 (Sigma) in 500 μ l of buffer C (20 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 150 mM KCl, 6 mM EDTA, 0.04% BSA) containing 0.1% Nonidet P-40 (NP-40) at 4°C for 1 h. Then, 30 μ l of protein A/G agarose beads (Santa Cruz Biotechnology) was added to the mixture and incubation was continued at 4°C for 3 h. Immune complex was pelleted by centrifugation, washed three times with buffer C containing 0.1% NP-40 and deproteinized by treatment with 1 mg/ml proteinase K and 1% SDS at 37°C for 1 h. Viral DNA was recovered by phenol/chloroform extraction and ethanol precipitation, suspended in TE containing 20 μ g/ml RNase and detected by Southern blotting analysis using ^{32}P -labeled probe for MoMLV LTR sequence (Lee and Craigie, 1994).

Preparation of bacterially expressed proteins

Human BAF protein was expressed and purified from *Escherichia coli* as described previously (Zheng *et al*, 2000). For purification of GST-fusion proteins, the cDNA encoding full-length LAP2 α (GST-LAP2 α) and its deletion mutants, GST-LAP2c (LAP2 common domain), GST-LAP2 α Δ N186 (LAP2 α -specific domain, residues 187–692), GST-LAP2 α Δ C253 (residues 1–439) and GST-LAP2 α Δ N112 (residues 113–692) were amplified by PCR and cloned into the *SmaI* site of pGEX-2T vector (Amersham-Pharmacia) and the recombinant plasmids were transformed into *E. coli*, strain BL21 (DE3) (Stratagene). An overnight culture from a single colony was diluted 1:50 in fresh LB medium containing 100 μ g/ml ampicillin, incubated at 37°C and, upon reaching an OD₆₀₀ of 0.5, protein expression was induced under the control of T7 RNA polymerase by addition of isopropyl β -D-thiogalactoside (IPTG) to 1 mM. Bacteria were harvested 5 h after induction and frozen in 1/10 volume of suspension buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA). The cells were then thawed on ice, lysed by addition of 0.4 mg/ml lysozyme, 0.028% β -mercaptoethanol and proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 5 μ g/ml aprotinin (Sigma)) at 4°C for 1 h and centrifuged at 30 000 r.p.m. for 1 h in a Beckman 45 Ti rotor. GST-fusion protein was bound to a glutathione Sepharose 4B column (Amersham-Pharmacia) and washed with phosphate-buffered saline (PBS) containing 500 mM NaCl, eluted with protein buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% (wt/vol) glycerol) containing 15 mM reduced glutathione

(Sigma). Fractions containing GST-fusion protein were further purified by gel filtration on a Superdex 200 column (Amersham-Pharmacia) in the same buffer and stored at -80°C .

To prepare His-tagged protein, the cDNAs of full-length LAP2 α (His-LAP2 α) and LAP2 α -specific domain (His-LAP2 α /ΔN186) were amplified by PCR and cloned into pET15b vector (Novagen) that had been digested with *Nde*I, and treated with Mung Bean Nuclease (New England BioLabs) for blunt end ligation. Expression of protein and lysis of bacteria were carried out by the same procedure as for GST-fusion proteins. The soluble fraction containing His-LAP2 α was loaded onto a Ni^{2+} -affinity chromatography column (Amersham-pharmacia) and the column was washed with PBS containing 500 mM NaCl and 20 mM imidazole. His-tagged LAP2 α was eluted by a 60–1000 mM imidazole gradient in PBS containing 500 mM NaCl and 10% glycerol and further purified by gel filtration on a Superdex 200 column equilibrated with protein buffer.

For the purification of His-LAP2 α /ΔN186, which was mostly found in insoluble fraction, the pellet after lysis was solubilized by 1/20 of original culture volume of urea buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 7 M urea) and incubated at room temperature for 30 min. After homogenization, the lysate was centrifuged at 30,000 r.p.m. for 1 h in a Beckman 45 Ti rotor and the supernatant was then loaded onto a Ni^{2+} affinity column. The His-tagged LAP2 α -specific domain protein was washed with PBS containing 500 mM KCl and 1 M urea, and eluted with a 60–1000 mM imidazole gradient in PBS containing 500 mM NaCl, 10% glycerol and 1 M urea. Fractions containing the His-LAP2 α /ΔN186 were pooled and dialyzed against the protein buffer containing 1 M urea.

Immunoprecipitation of PICs with anti-LAP2 α antibody

The His-LAP2 α /ΔN186 purified under partially denaturing conditions was used as an antigen to generate a rabbit serum against LAP2 α . Immunizations and serum production were carried out by Washington Biotechnology, Inc. Anti-LAP2 α polyclonal IgG was purified from the antisera using MabTrap Kit (Amersham-Pharmacia).

For immunoprecipitation, PIC fraction I was gel-filtrated through a spin column of Sephacryl S-1000 Superfine equilibrated with buffer B containing 0.1% BSA (fraction II) and 20 μl of the fraction II was incubated with anti-LAP2 α polyclonal IgG (5 μl), anti-BAF rabbit serum (20 μl , Suzuki and Craigie, 2002), control rabbit IgG, or control rabbit serum in 500 μl of buffer C containing 0.5% NP-40 at 4°C for 1 h. After adding 30 μl of protein A/G agarose beads, incubation was continued at 4°C for 3 h and the immune complex was washed three times with buffer C containing 0.5% NP-40. Finally, viral DNA was extracted from the immune complex and detected by Southern blotting using ^{32}P -labeled probe for MoMLV LTR sequence.

GST-pulldown assay

A volume of 20 μl of PIC fraction II was incubated with 100 nM GST-LAP2 α , GST-LAP2c, GST-LAP2 α /ΔN186, or GST in 100 μl of buffer D (20 mM Hepes-NaOH, pH 7.5, 5 mM MgCl_2 , 400 mM KCl, 6 mM EDTA, 0.04% BSA, 40% Nycodenz, 10 mM $(\text{NH}_4)_2\text{SO}_4$) on ice. After 1 h incubation, 30 μl of glutathione Sepharose 4B beads (50% slurry equilibrated with PBS, Amersham-pharmacia) was added to the mixture and the incubation was continued on ice for 30 min. Beads were washed with buffer C containing 0.05% NP-40 three times and deproteinized with proteinase K and SDS. Viral DNA from captured PICs was isolated and detected by Southern blotting using ^{32}P -labeled probe for MoMLV LTR sequence.

PIC integration activity assay

A volume of 20 μl of PIC fraction II or salt-stripped PIC was incubated with 10 and 100 nM BAF or His-LAP2 α in 100 μl of buffer D on ice for 1 h (Lee and Craigie, 1998). Integration activity of the PICs was evaluated by the previously described integration activity assay (Lee and Craigie, 1994, 1998).

Velocity sedimentation assay

DNA substrates were $\Phi \times 174$ DNA linearized with *Xho*I or a circular form of $\Phi \times 174$ DNA (replicative form II, New England Biolabs). BAF/DNA complex, with or without GST-fusion LAP2 α proteins or its deletion mutants, was formed with 10 nM proteins and 0.1 pM substrate DNA in 100 μl of a reaction mixture (20 mM Hepes-NaOH, pH 7.5, 115 mM KCl, 100 ng/ml BSA, 5 mM DTT) at 30°C for 1 h. To check the salt stability of the nucleoprotein complex, KCl was added to a final concentration of 150 or 400 mM

and the mixture was incubated on ice for 1 h. Continuous sucrose gradients (2 ml) were made by layering 15, 20, 25 and 30% sucrose solutions in buffer A containing KCl (150 or 400 mM) and 6 mM EDTA at 4°C overnight. The gradient was overlaid with the salt-challenged reaction (100 μl) mixture and centrifuged at 30 000 r.p.m. at 4°C for 1 h in a Beckman TLS55 rotor. The gradient was fractionated from the top into 21 fractions and each fraction was deproteinized by proteinase K and SDS. The DNA in each fraction was isolated by phenol/chloroform extraction and ethanol precipitation, and detected by Southern blotting using ^{32}P -labeled probe for $\Phi \times 174$ DNA sequence.

Establishment of LAP2 α -knockdown cell lines and isolation of PICs

A 19-bp siRNA sequence was selected from nucleotide positions 1063–1081 (5'-AGA GAA GUA CUG CAG GAG U-3') in the open reading frame of LAP2 α mRNA and synthesized DNA of this sequence was inserted into the pSilencer 2.1-U6 neo siRNA expression vector (Ambion) according to the manufacturer's protocol. As a negative control, we used the pSilencer 2.1-U6 neo Negative Control plasmid (Ambion), whose hairpin siRNA sequence is not found in the mouse genome database. The vectors were digested with *Xmn*I to linearize the plasmid DNA and transfected into NIH3T3 cells by the calcium phosphate transfection method. To establish stable siRNA-expressing cell lines, the transfected cells were selected and maintained in culture medium containing 800 $\mu\text{g}/\text{ml}$ neomycin (Invitrogen). Reduction in expression of LAP2 α protein expression was analyzed by western blotting using anti-LAP2 monoclonal antibody as described below. To isolate the PICs, 2×10^6 of cells were cultured with 10 ml of supernatant from clone 4 cells in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene for 5 h, and cytoplasmic extract was prepared from the infected cells using 500 μl of buffer A containing digitonin and stored in buffer B.

Western blotting analysis

Cells were trypsinized, washed with PBS and lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, 0.9% β -mercaptoethanol) by boiling. Samples were loaded on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred to Invitrolon PVDF membrane (Invitrogen). The blot was then subjected to immunoblotting using WesternBreeze chemiluminescent immunodetection kit (Invitrogen) and proteins were detected by a luminescence image analyzer, LAS 1000 (Fujifilm). Primary antibodies used for the western blotting analysis were anti-FLAG mouse monoclonal IgG (Clone M2, Sigma) for detection of FLAG-tagged proteins, anti-LAP2 α rabbit polyclonal IgG for detection of LAP2 α , anti-LAP2 mouse monoclonal IgG (Clone 27, BD Transduction Laboratories) for detection of LAP2 isoforms and anti-lamins A/C mouse monoclonal IgM (Clone 346, Santa Cruz Biotechnology).

Analysis of MoMLV replication

To prepare MoMLV virus stock, culture supernatant from clone 4 cells was harvested, filtered through a membrane (0.45 μm pore size) and stored at -80°C until use. The titer of the viral stock was determined by end-point titration of 10-fold dilution on cultured NIH3T3 cells. For an infection experiment, NIH3T3, LAP2 α -knockdown and siRNA control cell lines were exposed to 1×10^3 infectious virus particles per 2×10^5 cells in 60 mm diameter plates with culture medium containing 8 $\mu\text{g}/\text{ml}$ polybrene at 37°C for 2 h. After washing twice with fresh medium to remove residual free virus, the infected cells were maintained in culture medium and split 1:5 every 3 days. Virus production in culture supernatant from the MoMLV-infected cells at 3, 6 and 9 days after infection was monitored by an exogenous RT activity assay using ^{32}P -labeled dTTP as described previously (Telesnitsky *et al*, 1995).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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